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Formation of the Productive ATP-Mg²⁺-bound Dimer of GlcV, an ABC-ATPase from *Sulfolobus solfataricus*

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The ABC-ATPase GlcV from *Sulfolobus solfataricus* energizes an ABC transporter mediating glucose uptake. In ABC transporters, two ABC-ATPases are believed to form a head-to-tail dimer, with both monomers contributing conserved residues to each of the two productive active sites. In contrast, isolated GlcV, although active, behaves apparently as a monomer in the presence of ATP-Mg²⁺, AMPPNP-Mg²⁺ or ATP alone. To resolve the oligomeric state of the active form of GlcV, we analysed the effects of changing the putative catalytic base, residue E166, into glutamine or alanine. Both mutants are, to different extents, defective in ATP hydrolysis, and gel-filtration experiments revealed their dimerization in the presence of ATP-Mg²⁺. Mutant E166Q forms dimers also in the presence of ATP alone, without Mg²⁺, whereas dimerization of mutant E166A requires both ATP and Mg²⁺. These results confirm earlier reports for other ABC-ATPases, but for the first time suggest the occurrence of a fast equilibrium between ATP-bound monomers and ATP-bound dimers. We further mutated two highly conserved residues of the ABC signature motif, S142 and G144, into alanine. The G144A mutant is completely inactive and fails to dimerize, indicating an essential role of this residue in stabilizing the productive dimeric state. Mutant S142A retained considerable activity, and was able to dimerize, thus implying that the interaction of the serine with ATP is not essential for dimerization and catalysis. Furthermore, although the E166A and G144A mutants each alone are inactive, they produce an active heterodimer, showing that disruption of one active site can be tolerated. Our data suggest that ABC-ATPases with partially degenerated catalytic machineries, as they occur *in vivo*, can still form productive dimers to drive transport.

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Keywords: ABC-ATPase; ABC signature motif; dimerization; catalytic mechanism; catalytic base

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Introduction

In the hyperthermophilic archaeon *Sulfolobus solfataricus* an ABC transporter mediates glucose uptake.¹ This transporter is a multi-subunit complex similar to the histidine permease from *Salmonella typhimurium* and the maltose ABC transport system from *Escherichia coli*.^{2,3} It consists of an extracellular, membrane-anchored, glucose-bind-

ing protein (GlcS), two membrane-spanning proteins (GlcT, GlcU) that form a channel, and two copies of a cytosolic ATP-binding cassette (or ABC-ATPase), GlcV, which energize transport. The amino acid sequence of GlcV (353 residues; 40 kDa) features a number of motifs conserved in all ABC-ATPases. The Walker A and B motifs are common to many nucleotide tri-phosphate hydrolases,⁴ and contain residues that bind ATP and Mg²⁺, the catalytic cofactor. The ABC signature motif, however, is strictly specific to ABC-ATPases and includes residues essential for ATP hydrolysis, but not for ATP binding.^{5,6} The crystal structures of several ABC-ATPases revealed that these proteins

Abbreviations used: ABC, ATP-binding cassette; AMPPNP, adenosine 5'-(β,γ-imido)triphosphate.

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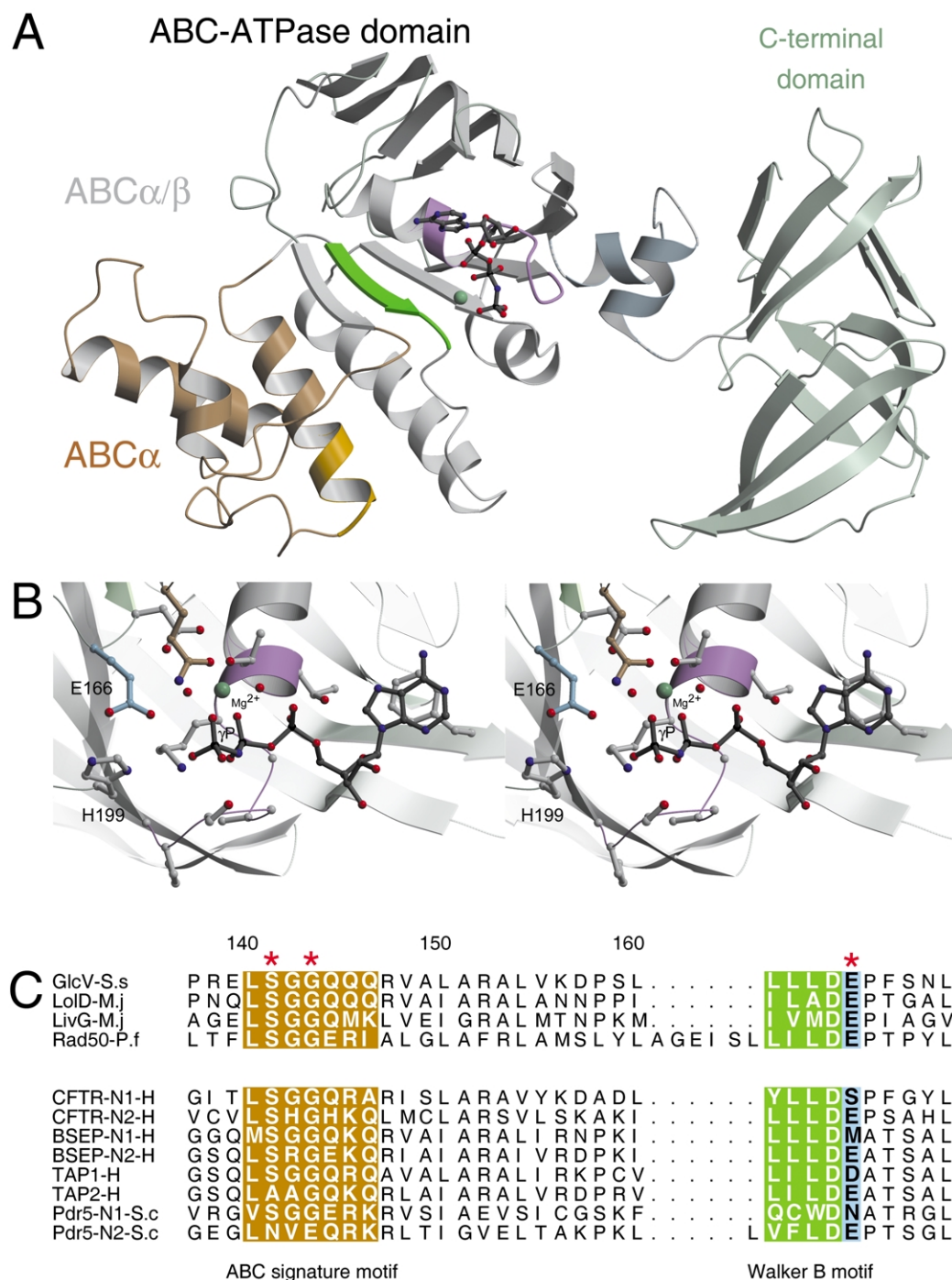


Figure 1. A, Ribbon representation of the three-dimensional structure of GlcV from *Sulfolobus solfataricus*. The locations of conserved ABC-ATPase motifs are indicated by different colours: Walker A motif, purple; Walker B motif, green; ABC signature motif, yellow. Also shown is the location of bound AMPPNP-Mg²⁺.¹³ B, Stereo view of the nucleotide-binding site in the GlcV-AMPPNP-Mg²⁺ structure.¹³ Residue side-chains, the nucleotide, the Mg²⁺, and its coordinating water molecules are shown in ball-and-stick representation. Residue E166, the putative catalytic base, is coloured in blue. C, Part of a sequence alignment of GlcV from *Sulfolobus solfataricus* with the ABC-ATPases LivG (LivG-M.j) and LolD (LolD-M.j) from *Methanocaldococcus janasschii*, the ABC-type ATPase domain of Rad50 from *Pyrococcus furiosus* (Rad50-P.f), and the ABC-ATPase domains of CFTR from human (CFTR-N1-H; CFTR-N2-H), BSEP from human (BSEP-N1-H; BSEP-N2-H), TAP from human (TAP1-H; TAP2-H) and Pdr5 from *Saccharomyces cerevisiae* (Pdr5-N1-S.c; Pdr5-N2-S.c). Residue numbers refer to GlcV. The residues mutated in GlcV are indicated with a red asterisk.

share a common fold and nucleotide binding site configuration.^{7–12} Recently, we have determined high-resolution crystal structures of GlcV,¹³ in nucleotide-free states, and as complexes with bound ADP-Mg²⁺ and AMPPNP-Mg²⁺ (Figure 1). The structure of GlcV consists of a typical ABC-ATPase domain and a C-terminal domain of unknown function. Residues of the Walker A and B motifs are located in the ABC α / β -subdomain, and form most of the nucleotide-Mg²⁺ binding site, while the ABC signature residues are present in the ABC α -subdomain, at about 25 Å from the nucleotide-Mg²⁺ binding site. The different crystal structures of GlcV are all of monomeric protein, in agreement with the behavior of GlcV during size-exclusion chromatography, both in the absence of nucleotides and in the presence of ADP-Mg²⁺, ATP-Mg²⁺, AMPPNP-Mg²⁺ or ATP alone.

The crystal structure of the ATPase domain of Rad50 from *Pyrococcus furiosus*, involved in DNA maintenance, showed that ABC-ATPase domains occur in other mechano-chemical systems.¹⁴ Its structure in complex with AMPPNP-Mg²⁺ revealed a head-to-tail dimer, in which two functional active sites form at the dimer interface, each by juxtaposition of ABC signature motif residues of one monomer and the nucleotide-Mg²⁺ binding residues of the other monomer. Biochemical analysis of the *E. coli* maltose ABC transporter¹⁵ and the structure of the transporter complex BtuCD¹⁶ established this head-to-tail arrangement as the archetype of the productive dimeric state of all ABC-ATPases. It explains the positive cooperativity in ATP hydrolysis that is observed for the ABC-ATPases in uptake systems,^{17,18} and the negative effect of mutations in the ABC signature motif on ATP hydrolysis and transport activities.^{5,6} Moreover, an association/dissociation cycle of the two ABC-ATPases, coupled to ATP binding and hydrolysis, could be part of a mechanism that explains the energy-transducing properties of ABC transporters.^{14,19} Evidence for ATP-controlled dimerization of ABC-ATPases in transporters comes from studies on isolated forms of these proteins, in particular LolD and LivG from *Methanocaldococcus jannaschii*.^{20,21} Like GlcV, wild-type LolD and LivG elute as monomers during size-exclusion chromatography, in the absence and in the presence of ATP. However, mutation of the putative catalytic base (E171 in LolD, E179 in LivG) to either glutamine or alanine produced ABC-ATPases that form stable dimers in an ATP-dependent manner.²⁰ Since the mutants could still bind, but no longer efficiently hydrolyze ATP, the different behaviors of the wild-type and mutant proteins were explained by the existence of a short-lived ATP-dependent dimeric state in the catalytic cycle of these transport ABC-ATPases. The structural similarity of this dimer with the head-to-tail dimer of Rad50 was confirmed by X-ray crystallography.²¹

Wild-type GlcV is catalytically active in solution, in the presence of Mg²⁺, with an optimal activity at pH 6.5. Considering the high level of structural

similarity of its ABC-ATPase domain with that of other ABC-ATPases, including LolD and LivG, we expect that the catalytic cycle of GlcV involves a transient ATP-bound dimeric state. On the other hand, the presence of a C-terminal domain in GlcV, which is absent from LolD and LivG, could have some unexpected effect on its behavior. Therefore, to resolve the oligomeric state of the ATP-Mg²⁺-bound, productive form of GlcV, we took an approach similar to that used for LolD and LivG, analysing single mutants that are able to bind ATP, but which are defective in ATP hydrolysis. Residue E166 in GlcV, located just after the Walker B motif, is the equivalent of E171 and E179 in LolD and LivG, respectively (Figure 1C), and was mutated to a glutamine or alanine residue. Furthermore, we mutated the most highly conserved ABC signature residues (S142A, G144A). We studied the effects of these mutations on the ATPase activity and the oligomeric state of GlcV by means of ATPase activity assays and gel-filtration experiments. Our results indicate strongly that ATP binding induces dimerization of GlcV. Moreover, they reveal an unexpected tolerance of its ABC-ATPase domain to modifications of the catalytic machinery with respect to its ability to form a dimer and hydrolyze ATP.

Results and Discussion

ATPase activity of the GlcV mutants

The GlcV mutants were isolated to near-homogeneity, as judged from silver-stained SDS-PAGE analysis (Figure 2(a)). ATPase activity assays revealed that all the mutants are, to different extents, defective in ATP hydrolysis (Figure 2(b)), although they bind ATP, as deduced from elution profiles recorded during gel-filtration experiments performed in the presence of ATP-Mg²⁺ (see below). The E166Q mutant lost about 80% of the wild-type activity, while no significant activity was measured for mutant E166A. These substantial losses of activity, particularly of the E166A mutant, emphasize the important role of residue E166 in ATP hydrolysis, most probably as a catalytic base. The role of residue E166 as a catalytic base in GlcV agrees with the GlcV-AMPPNP-Mg²⁺ structure (Figure 1B),¹³ in which it is the only acidic residue near the γ -phosphate group in a suitable position to activate a water molecule. The rather high residual activity of the E166Q mutant is surprising, but shows that this highly conserved glutamate residue is not essential for ATP hydrolysis. A similar residual activity was reported recently for the equivalent mutant of the nucleotide-binding domain of Mdl1p from *Saccharomyces cerevisiae*.²² A possible explanation is that in these mutants the function of catalytic base is taken over partly by another residue. The most likely candidate for such a back-up role in GlcV-E166Q is the highly conserved H-motif histidine residue

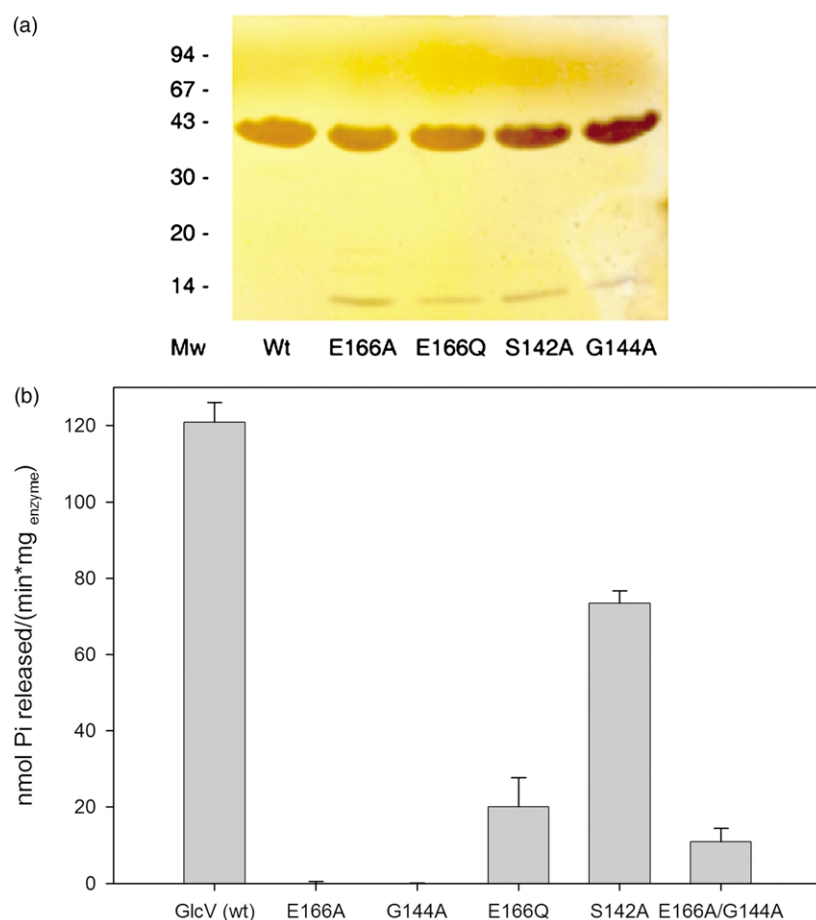


Figure 2. (a) Silver-stained SDS-PAGE analysis of purified wild-type GlcV and mutants E166A, E166Q, S142A and G144A (this work): Mw, molecular mass markers (kDa); Wt, wild-type GlcV. (b) ATPase activity of wild-type GlcV, the E166A, G144A, E166Q, and S142A mutants, as well as of an equimolar mixture of mutants E166A and G144A.

(H199), which, like E166, is positioned near the γ -phosphate group in the GlcV-AMPPNP- Mg^{2+} structure (Figure 1B). In the Rad50-AMPPNP- Mg^{2+} structure the equivalent histidine residue was found to stabilize the putative catalytic water molecule in front of the γ -phosphate group.¹⁴ However, additional data on the catalytic and thermodynamic properties of GlcV-E166Q (e.g. the pK_a value of residue 199) are required to verify a backup role of H199 as catalytic base. The complete loss of ATPase activity exhibited by the E166A mutant suggests that in this mutant the catalytic water molecule can no longer be positioned properly or stabilized.

The mutations in the ABC signature motif result in significant losses of ATPase activity, about two-fold for the S142A mutant, while the G144A mutant is completely inactive. To assess the cause of this complete loss of ATPase activity, we have determined the crystal structure of the G144A mutant at high resolution (Table 1). Comparison with the wild-type GlcV structure shows that the methyl group introduced in residue 144 did not lead to any conformational changes in the ABC signature motif loop (Figure 3), nor to any changes in the nucleotide-binding site. Likewise, we do not expect that the lower ATPase activity of the S142A mutant is caused by structural changes to GlcV in its monomeric form, since the crystal structures

of wild-type GlcV show that the side-chain of this serine residue is fully exposed to the solvent, and not involved in any interaction. Considering also the large distance of over 25 Å between the ABC signature motif and the nucleotide-binding site observed in the structures of monomeric GlcV, these results strongly suggest that residues S142 and G144 participate in ATP binding and/or hydrolysis *via* an oligomeric arrangement of GlcV.

ATP-dependent dimerization of the E166Q and E166A mutants

Size-exclusion chromatography was used to determine the oligomeric state of wild-type GlcV and the four mutants (Figure 4(a)). In the absence of nucleotide- Mg^{2+} , the variants eluted with an apparent molecular mass of about 40 kDa, corresponding to the monomer. For the wild-type protein, the retention volume remained the same in the presence of ATP- Mg^{2+} , as well as in the presence of ADP- Mg^{2+} or ATP without Mg^{2+} (data not shown). Under these conditions, nucleotide is bound to the protein, as indicated by the increased ratio of the absorbance measured at 254 nm and 280 nm (A_{254}/A_{280}), compared to an experiment performed in the absence of nucleotide. Nucleotide binding was observed for mutants E166Q and E166A. However, while both mutants still eluted

Table 1. Crystallographic data of GlcV-G144A

<i>A. Data collection</i>	
Beamline	ID14-EH2, ESRF
Detector	ADSC Q4 CCD
Temperature (K)	100
<i>B. Data processing and reduction</i>	
Space group	$P2_12_12_1$
Unit-cell dimensions	
<i>a</i> (Å)	46.3
<i>b</i> (Å)	48.4
<i>c</i> (Å)	183.5
Number of molecules in the a.u.	1
Number of unique reflections	62,479
Average redundancy	2.93
Resolution range (Å)	33.5–1.45
Completeness (%) ^a	80.0 (47.1) (31.2)
$\langle I/\sigma(I) \rangle$ ^a	14.3 (2.5) (2.0)
R_{sym} (%) ^{a,b}	7.5 (26.6) (37.3)
<i>C. Refinement</i>	
Resolution range (Å)	33.5–1.50
$R_{\text{factor}}/R_{\text{free}}$ (%) ^c	17.2/21.2 (20.6/25.7)
Number of atoms	3483
Number of non-protein atoms	731
rmsd bond lengths (Å)	0.009
rmsd bond angles (°)	1.4
Average <i>B</i> all atoms (Å ²)	18.0
Average <i>B</i> water molecules (Å ²)	37.3
<i>D. Residues in Ramachandran plot</i>	
Most favored regions (%)	92.8
Allowed regions (%)	6.9
Generously allowed regions (%)	0.3
Disallowed regions (%)	0.0

^a In parentheses are the values for the two highest-resolution shells, 1.55–1.50 Å and 1.50–1.45 Å.

^b $R_{\text{sym}} = \sum_h \sum_i |I_{(h,i)} - \langle I_h \rangle| / \sum_h \sum_i \langle I_h \rangle$.

^c $R = \sum_h \|F_o\| - \|F_c\| / \sum_h \|F_o\|$. In parentheses are the values for the highest-resolution shell, 1.55–1.50 Å.

as monomers in the presence of ADP-Mg²⁺, they eluted with an apparent molecular mass of about 60 kDa in the presence of ATP-Mg²⁺. Hence, we conclude that this shift toward a higher molecular mass is correlated with ATP binding and that it demonstrates the formation of dimers in an ATP-dependent manner. Although this molecular mass

of 60 kDa differs from the 80 kDa expected for a GlcV dimer, the increase of 20 kDa in molecular mass is too large to be caused by a conformational change in the monomer. This molecular mass of 60 kDa can be explained by the occurrence of a fast equilibrium between monomeric and dimeric states, and a rather high dissociation constant of the dimer (K_d) in the micromolar range. For mutant E166Q this apparent instability of the dimer may be explained by its residual ATPase activity, as the ABC-ATPase dimer is expected to dissociate upon ATP hydrolysis.^{20,21} However, ATP hydrolysis cannot be the only factor governing the monomer–dimer equilibrium, since mutant E166A, although inactive, behaves similarly to mutant E166Q during gel-filtration in the presence of ATP-Mg²⁺. Furthermore, in the presence of ATP alone (without Mg²⁺), when no ATPase activity is observed, mutant E166Q still shows an apparent molecular mass of about 60 kDa (Figure 4(b)). At low or moderate concentrations of protein (12.5–50 μM), ATP-dependent dimerization of GlcV was observed only if ATP was present in the mobile phase of the gel-filtration column. This behavior differs from that reported for LolD²⁰ and indicates that the ATP-bound GlcV dimers are relatively unstable, consistent with the difference in K_m for ATP hydrolysis observed for these proteins (290 μM and 50 μM for GlcV²³ and LolD,²⁰ respectively). In contrast, at high concentrations of protein (125–250 μM) our gel-filtration assay revealed dimerization of the E166Q mutant without ATP being present in the mobile phase. This behavior agrees with the existence of a fast monomer–dimer equilibrium with the protein monomers having a relatively weak binding affinity for ATP. We propose that at low concentrations of GlcV, dissociation of ATP from the GlcV monomers is faster than the association of two ATP-bound monomers forming a dimer, while at higher concentrations of protein the rate of dimer formation is faster, explaining the increase in stability of the ATP-bound GlcV dimer for increasing concentrations of

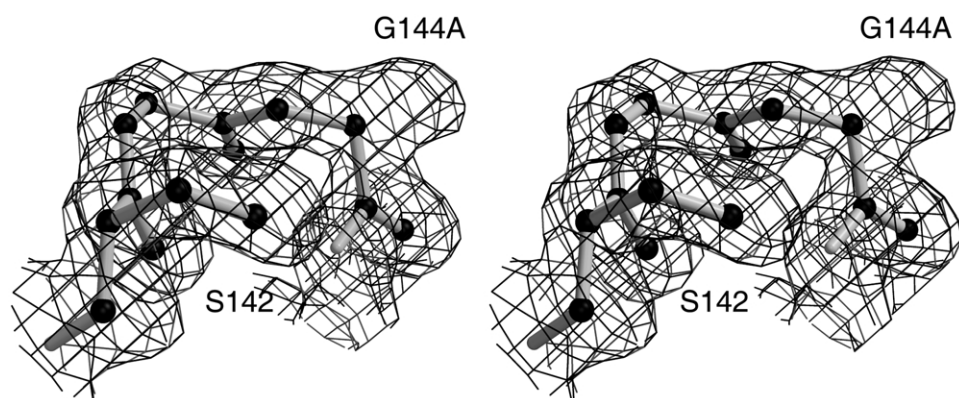


Figure 3. Stereo view of electron density around residue 144 of the ABC signature motif calculated for the G144A structure at 1.5 Å resolution. Superimposed on the density is the atomic structure of wild-type GlcV. The electron density is part of a simulated annealing $2F_o - F_c$ omit map calculated using CNS³⁷ after first omitting residues 135–153 from the model. The map was contoured at 1.0σ .

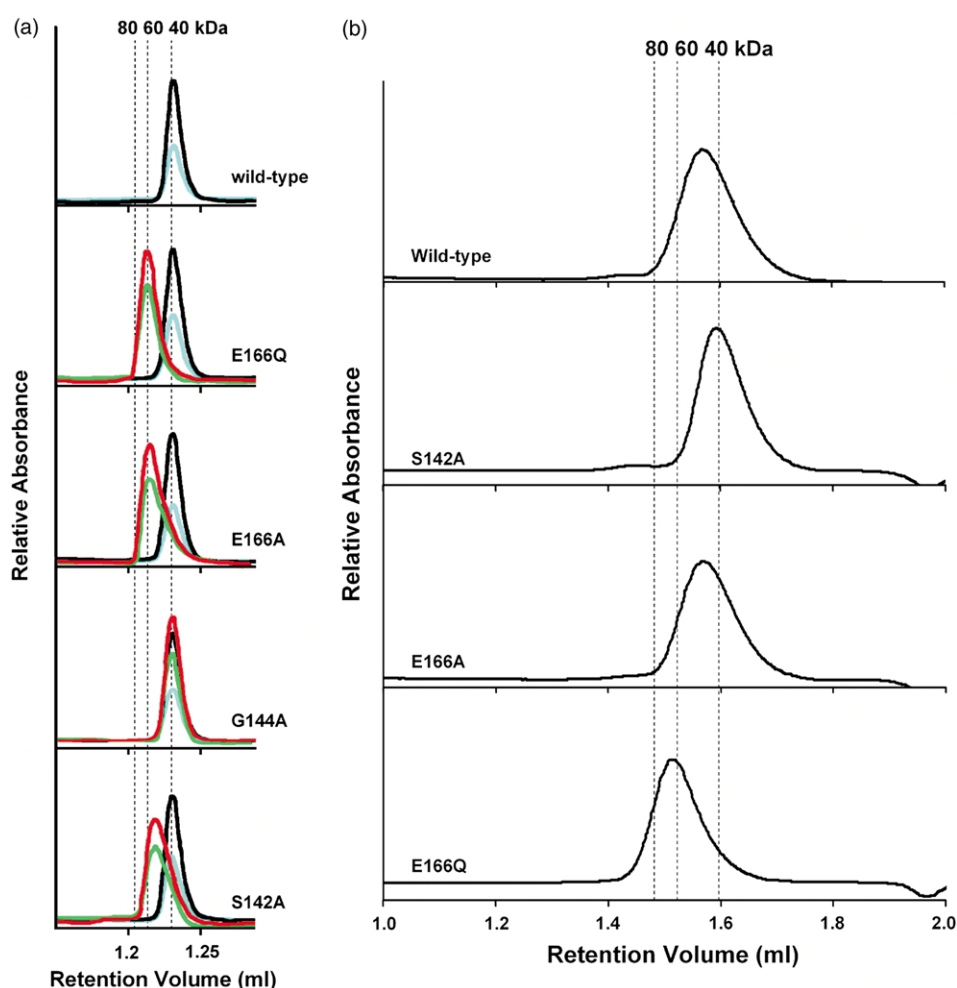


Figure 4. (a) Elution profiles recorded during gel-filtration experiments performed for wild-type GlcV and mutants E166Q, E166A, G144A, and S142A. These experiments were performed in the absence of nucleotide- Mg^{2+} (recorded at 280 nm (black) and at 254 nm (blue)) and in the presence of ATP- Mg^{2+} (recorded at 280 nm (red) and at 254 nm (green)) using a Superdex 75 column and protein concentrations of 0.5 mg/ml (12.5 μ M). ATP was present in the equilibration and elution buffer at 0.1 mM. Particular elution volumes (ml) are indicated on a horizontal axis. (b) Gel-filtration elution profiles for wild-type GlcV, and mutants E166Q, E166A and S142A, in the presence of 0.5 mM ATP and 2 mM EDTA, both in the sample buffer and in the column buffer. The protein concentrations were about 0.250 mM. A Superdex 200 column was used instead of a Superdex 75 column.

protein. To investigate this possibility further, a titration experiment was performed by loading samples of GlcV-E166Q onto the gel-filtration column with a fixed, high concentration of protein (0.25 mM), but a variable concentration of ATP (Figure 5). The mobile phase of the gel-filtration column contained 2 mM EDTA, but no ATP. With a twofold molar excess of ATP, the elution profile shows two peaks, one representing free ATP and one representing ATP-bound GlcV-E166Q involved in dimerization with an average molecular mass of 60 kDa. With a concentration of ATP that equals the concentration of GlcV-E166Q, the elution profile still shows the 60 kDa peak, but the peak for free ATP has nearly disappeared, thus revealing that almost all the nucleotide is bound to the protein. At a concentration of ATP that is half that of the protein, two peaks are observed: one corresponding to 60 kDa, the other to 40 kDa, i.e. some

of the protein is involved in dimerization, while the remainder elutes as a monomer. The A_{254}/A_{280} ratio of the 40 kDa peak is significant smaller than that of the 60 kDa peak, indicating that the nucleotide is associated mainly with the protein that is involved in dimerization. Together, these results suggest strongly that, at least in the absence of Mg^{2+} and at relatively high concentrations of protein, a fast equilibrium occurs between monomeric ATP-bound and dimeric ATP-bound states of GlcV-E166Q, with an ATP binding stoichiometry of 2:1 relative to the GlcV dimers.

Behavior of the S142A and G144A mutants in size-exclusion chromatography

In contrast to the E166Q mutant, the G144A mutant always behaves as a monomer during gel-filtration, in the absence or in the presence of

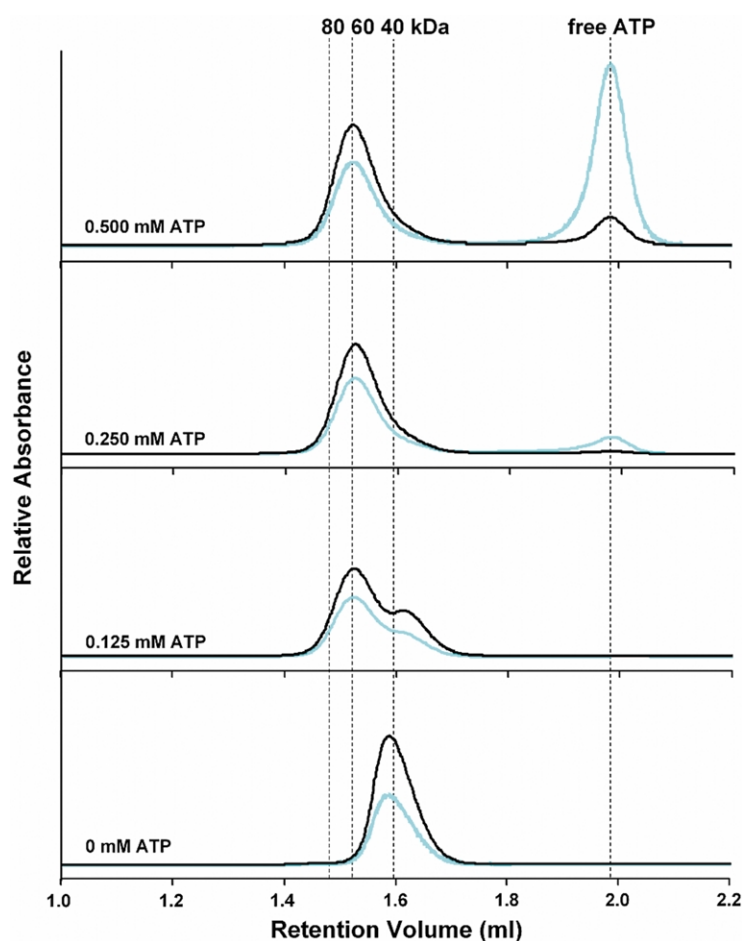


Figure 5. Elution profiles recorded for different gel-filtration runs on a Superdex 200 column loading a fixed amount of GlcV-E166Q (0.250 mM in 20 μ l) with different concentrations of ATP. The concentration of EDTA (in sample and column buffer) was 2 mM. No ATP was present in the column buffer. Absorbance was measured at 280 nm (black curves) and 254 nm (blue curves).

ATP-Mg²⁺ (Figure 4(a)), as well as in the presence of ATP alone (not shown). ATP does bind to mutant G144A, as shown by a higher A_{254}/A_{280} ratio compared to an experiment performed in the absence of nucleotide. Mutant S142A, on the other hand, shows an apparent molecular mass between those of the E166 mutants and wild-type GlcV (Figure 4(a)), all measured in the presence of ATP-Mg²⁺. The intermediate molecular mass correlates with the residual ATPase activity of this mutant, which is also between those of mutant E166Q and the wild-type protein. The behavior of the S142A and G144A mutants is consistent with a head-to-tail arrangement of the ATP-bound ABC-ATPase dimer, in which these residues participate in ATP binding across the dimer interface, *via* hydrogen-bonding contacts between their side-chain hydroxyl and main-chain amide groups, respectively, and one oxygen atom of the γ -phosphate group of ATP (Figure 6).^{14,21} Consistent with its elution as a monomer in the presence of ATP and Mg²⁺, a superposition of the G144A structure on that of the LolD dimer suggests that the alanine residue at position 144 in GlcV causes steric hindrance to one oxygen atom of the γ -phosphate group of ATP, as well as to the side-chain of S40 in the P-loop of the opposing monomer, and thus destabilizes the dimer interface (Figure 6B). The complete loss of ATPase activity exhibited by

the G144A mutant also suggests strongly that only the dimeric form of GlcV is active, since the crystal structure of monomeric G144A did not reveal any conformational changes compared to the wild-type structure of GlcV. Furthermore, considering also the significant residual ATPase activity of the S142A mutant, it is evident that the interaction of the main-chain amide group of the glycine residue with one oxygen atom of the ATP γ -phosphate group is essential and sufficient to form a productive dimer. In contrast, the interaction of the hydroxyl group of the serine with the γ -phosphate group of ATP is not absolutely essential for catalysis and/or dimerization.

Comparison with other ABC-ATPases

Our observations are consistent with GlcV forming transient dimers in the presence of ATP-Mg²⁺, as was shown for the transport ABC-ATPases LivG and LolD,²⁰ as well as for the nucleotide-binding domain of Mdl1p from *Saccharomyces cerevisiae*.²² The rather low stability of the ATP-bound GlcV dimer is surprising, as it deviates significantly from the results obtained for other ABC-ATPases. This difference may be related to the extra C-terminal domain in GlcV that is absent from LivG, LolD and the nucleotide-binding domain of Mdl1p. In a model of the GlcV dimer,¹³

built by using the LolD dimer structure as a template, the C-terminal domains are in close proximity to each other, indicating that they may well influence dimer formation. A better understanding of the interactions that stabilize the GlcV dimer, however, has to await crystallographic determination of the ATP-bound GlcV-E166Q dimeric structure.

The ability of GlcV-E166Q to form dimers in the presence of ATP alone, without Mg^{2+} , is similar to that reported for the equivalent mutants of LolD and the nucleotide-binding domain of Mdl1p. The crystal structure of the ATP-bound dimer of LolD-E171Q revealed a sodium ion occupying the Mg^{2+} site, which is believed to help stabilizing the bound ATP and to balance the electrostatic charge in the nucleotide-binding site, two necessary requirements for stable dimer formation.^{20,21} Since our gel-filtration assay contained 0.2 M NaCl, it is conceivable that, in the absence of Mg^{2+} , dimerization of GlcV-E166Q is stabilized by binding of ATP- Na^+ . The lack of ATP-induced dimer formation observed for GlcV-E166A in the absence of Mg^{2+} , indicates that in this mutant there is no stable binding of a sodium ion in the Mg^{2+} -binding site. This may be understood from the additional role of the conserved glutamate residue in binding the Mg^{2+} in the wild-type protein, as evident from the various structures of nucleotide-bound ABC-ATPases, including that of AMPPNP- Mg^{2+} -bound GlcV.¹³ While a glutamine residue, replacing the glutamate, can still participate in metal binding, an alanine residue cannot, and the E166A mutation is therefore expected to weaken the binding of metal ions to the Mg^{2+} -binding site. Wild-type GlcV requires Mg^{2+} for activity, and our gel-filtration assay shows only a very limited degree of dimerization in the presence of ATP- Na^+ . Similarly, no dimerization was observed for mutant S142A in the presence of ATP- Na^+ . These results are similar to those obtained for wild-type LolD²⁰ and further substantiate the notion that a proper balance of the electrostatic charge in the nucleotide-binding site of a transport ABC-ATPase is essential to stabilize the ATP-bound dimer.

GlcV mutants E166A and G144A form a catalytically active heterodimer

To provide further evidence that the productive, dimeric state of GlcV has a head-to-tail arrangement, we performed ATPase activity measurements with a sample containing equal amounts of mutants E166A and G144A. As presented in Figure 2(b), the E166A/G144A mixture exhibited substantial ATPase activity, about 12-fold lower than that of the wild-type protein. Since the mutants alone are inactive, the ATPase activity measured for this mixture can be explained only by the formation of a head-to-tail heterodimer of the ATP- Mg^{2+} -bound E166A and G144A mutants. In this heterodimer, the two inactivating mutations coincide in one active site, while the other active site carries no

mutations and is still functional. The ability of mutants E166A and G144A to form a heterodimer is surprising, considering the structure of the ATP- Na^+ -bound LolD dimer and the results of our gel-filtration experiments with GlcV-G144A. It is expected that in the E166A/G144A heterodimer the steric hindrance caused by the introduced methyl group in mutant G144A destabilizes the dimer interface significantly, and probably prevents formation of inter-subunit contacts with ATP- Mg^{2+} bound at the binding site of mutant E166A. The ATPase activity measured for the E166A/G144A mixture, which, after correcting for the occurrence of inactive ATP- Mg^{2+} -bound E166A homodimers, equals at most 20% of the wild-type activity, reflects the low level of stability of the E166A/G144A heterodimer. Nevertheless, the inter-subunit contacts formed at the second ATP- Mg^{2+} -binding site in the heterodimer apparently provide sufficient stabilization for ATP hydrolysis to occur.

Our mixing experiment reveals that in the productive dimer of GlcV, at least in its isolated form, the two active sites do not both need to be functional. In accordance with this observation, no cooperativity in ATPase activity could be detected for GlcV (our unpublished results). It should be noted, though, that limitations of our ATPase assay preclude measurements of accurate activities at low concentrations of ATP and at high concentrations of enzyme. Also, the properties of GlcV in our experiment are likely to be influenced by its unnatural environment, which differs significantly from that in the full transporter. Unfortunately, it is not possible to verify the effects of the mutations on the catalytic properties of GlcV in the full transporter, since our efforts to reconstitute this glucose ABC transporter from *Sulfolobus solfataricus* have so far been unsuccessful. For certain ABC transporters, like the maltose transport system and the P-glycoprotein multi-drug resistance pumps, it has been shown that hydrolysis of ATP at the two nucleotide-binding sites of the ABC-ATPase dimer is highly cooperative, and requires two fully intact ABC-ATPase domains.^{17,24–26} On the other hand, there are ABC transport systems in which the ABC-ATPases are quite tolerant to certain mutations in their catalytic machinery. For instance, for HisP, the ABC-ATPase of the histidine permease,² it has been shown that with only one functional active site the ABC-ATPase dimer is still able, to some extent, to energize transport.²⁷ Furthermore, *in vivo* a number of ABC transporters occur, in which one or both ABC-ATPases present a partially degenerated catalytic machinery. For instance, in one of the two ABC-ATPase domains of the human TAP system, the ABC transporter associated with antigen processing,²⁸ the highly conserved glutamate residue, located just after the Walker B motif, is substituted by an aspartate residue, while in the other ABC-ATPase domain the highly conserved serine residue of the ABC signature motif is replaced by an alanine residue

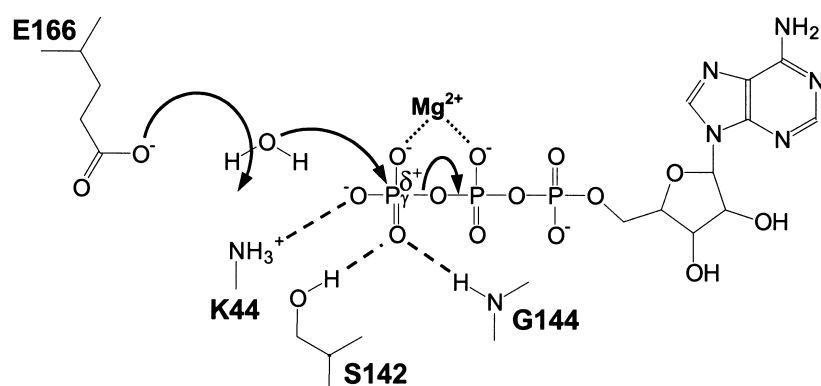


Figure 7. Proposed mechanism of ATP hydrolysis catalyzed by GlcV.

(Figure 1C). In CFTR, the cystic fibrosis transmembrane conductance regulator,²⁹ one ABC-ATPase domain presents a serine residue instead of the conserved glutamate residue, while in BSEP, a bile salt export pump,³⁰ the equivalent residue is methionine. For CFTR, there is strong evidence that the non-equivalency of the ABC-ATPase domains leads to two different roles for the composite active sites in the ABC-ATPase dimer: one is catalytically functional, while the other may have regulatory functions.³¹ Indeed, based on our results with GlcV, we would predict that these substitutions will not affect formation of the productive ABC-ATPase dimer, but will lead to differences in the catalytic efficiencies of the two active sites. This view is supported further by the results of studies of TAP, which show a remarkable difference in properties of its two ABC-ATPase domains with respect to nucleotide binding and ATP hydrolysis.^{32–34} There exist a number of ABC transporters, like Pdr5p from *Saccharomyces cerevisiae*,³⁵ in which one ABC-ATPase domain has the highly conserved serine and glycine residues of the ABC signature motif both replaced by more bulky residues (Figure 1C). On the basis of the behavior of mutant GlcV-G144A in our gel-filtration assay, and the structure superposition of GlcV-G144A on the ATP- Na^+ -bound LolD-E171Q dimer (Figure 6B), we expect these substitutions to seriously destabilize the inter-subunit contacts at one of the ATP-binding sites in the dimer. We thus propose that translocation in these transporters is driven by an ABC-ATPase dimer in which only one active site is formed.

Conclusions

Our analysis of the GlcV mutants strongly suggest that binding of ATP and Mg^{2+} to monomeric GlcV results in the formation of a productive dimer. Moreover, the behavior of mutants S142A and G144A, as well as the substantial ATPase activity of the E166A/G144A heterodimer are in full agreement with a head-to-tail arrangement of this dimer similar to that observed in the crystal structures of the ATP-bound dimers of Rad50 and LolD.¹⁴ In contrast to GlcV mutants E166A and

S142A, dimerization of GlcV-E166Q depends only on the presence of ATP, and not Mg^{2+} , probably because a sodium ion is able to bind to the Mg^{2+} -binding site and stabilize the ATP-bound E166Q dimer, as shown for the ATP-bound LolD-E171Q dimer. Unique to GlcV are the rather low level of stability of the ABC-ATPase dimer and the possible occurrence of a fast equilibrium between ATP-bound monomers and ATP-bound dimers, which may reflect an influence on dimerization of the extra C-terminal domain present in GlcV.

Our results provide evidence for a catalytic mechanism of ATP hydrolysis common to most ABC-ATPases (Figure 7). The highly conserved glutamate residue located just after the Walker B motif serves as the base that activates a nucleophilic water molecule. The serine and glycine residues of the ABC signature motif, together with the Mg^{2+} and residues of the Walker A motif, notably the highly conserved lysine residue, stabilize the negative charge that develops on the γ -phosphate group, thereby lowering the energy of the transition state and facilitating the productive nucleophilic attack of the activated water molecule on the ATP γ -phosphate group. Our data show that certain mutations of residues that compose this catalytic machinery, like the glutamate residue located just after the Walker B motif, and the highly conserved serine residue of the ABC signature motif can be tolerated with respect to the ability of an ABC-ATPase to form a dimer and hydrolyze ATP. Mutation of the highly conserved glycine residue of the ABC signature motif is not tolerated, unless it occurs in only one of the two ABC-ATPase subunits. Overall, our data suggest strongly that ABC-ATPases with partially degenerated catalytic machineries, as they occur *in vivo* or may appear through the course of evolution *via* certain mutations, can still form productive dimers to drive transport.

Materials and Methods

Expression and purification of GlcV mutants

Each mutation was introduced by PCR (Quick Change TM kit, Stratagene) into the *glcV* gene inserted into the

expression plasmid pET-15b (Novagen) and checked by sequencing. Mutated proteins were produced in *E. coli*, as described for the wild-type protein.²³ Cell extracts were prepared by sonication in buffer A (50 mM Mes (pH 6.5), 100 mM NaCl) and clarified by centrifugation at 40,000g for 30 minutes. For each extract, the supernatant was mixed with an equal volume of buffer A pre-heated at 90 °C, incubated immediately for 20 minutes at 70 °C, and re-clared by centrifugation at 120,000g for 60 minutes. Each mutated protein was purified using a Red-dye agarose column, as described.²¹ The purity of each preparation was assessed with a silver-stained SDS-PAGE analysis (Figure 2(a)).

ATPase activity measurements

For activity measurements, 3 µg of purified protein was pre-heated at 70 °C for two minutes in 100 µl of assay solution (25 mM Mes (pH 6.5), 150 mM NaCl, 5% (v/v) glycerol, 1 mM MgCl₂). Reactions were initiated by adding ATP (to 1 mM) and stopped after five minutes by freezing in liquid nitrogen. For measurements to be carried out in the absence of Mg²⁺, MgCl₂ was omitted from the assay solution and EDTA was added (to 3 mM). The amount of released inorganic phosphate was measured using a colorimetric method.³⁶ The data were corrected for non-enzymatic hydrolysis of ATP.

Gel-filtration experiments

Samples (20 µl) of purified protein (wild-type or mutants in 25 mM Mes (pH 6.5), 200 mM NaCl, 5% glycerol) were incubated overnight at room temperature under ATP-hydrolysing conditions (in the presence of MgCl₂) or non-hydrolysing conditions (without Mg²⁺, but with EDTA) with different concentration of nucleotides (ADP or ATP). Gel-filtration experiments were carried out using either a Superdex 75 or Superdex 200 column (PC 3.2/30; bed volume 2.4 ml) mounted on a SMART system (Amersham Biosciences). The columns were equilibrated and eluted at room temperature, using a flow-rate of 50 µl/minute, with the solution used to prepare the samples, including the nucleotide if indicated. The protein elution was monitored with the absorption profiles recorded at 254 nm and 280 nm. The molecular mass corresponding to an observed peak was deduced from the retention volumes measured for several molecular mass standards: γ-globulin, 158 kDa; albumin, 67 kDa; ovalbumin, 44 kDa; chymotrypsinogen A, 25 kDa; and ribonuclease A, 14 kDa.

Crystallization and structure determination of GlcV mutant G144A

Crystals of the G144A mutant were grown by micro-seeding from wild-type GlcV crystals in hanging-drop, vapour-diffusion set-ups, as described.²³ Diffraction data were collected at beamline ID14-EH2 (ESRF, Grenoble) to 1.45 Å resolution and reduced with the programs DENZO and SCALEPACK.³⁷ Structure factors were calculated using CCP4 programs.³⁸ Structure refinement was carried out with CNS,³⁹ using one of the GlcV nucleotide-free structures as a starting model.¹³ The refinement was monitored with an *R*_{factor} and an *R*_{free} set including 5% of the data.⁴⁰ Difference Fourier maps at the initial stage of the refinement showed that residue G144 was mutated to alanine. Rebuilding of the model was performed using the program QUANTA (Accelrys,

San Diego). Water molecules were placed using the program ARP/wARP.⁴¹ Final rounds of refinement were carried out with the program REFMAC5,⁴² including TLS refinement.⁴³ The quality of the model was assessed using the program PROCHECK.⁴⁴ Crystallographic data and statistics related to the data processing and the quality of the refined model are presented in Table 1.

Protein Data Bank accession code

The G144A mutant coordinates have been deposited at the Protein Data Bank⁴⁵ (code: 1OXX).

Miscellaneous

Structure Figures were prepared using MOLSCRIPT,⁴⁶ BOBSCRIPT⁴⁷ and RASTER3D.⁴⁸ The sequence alignment was generated using the program CLUSTALW⁴⁹ at the EBI server† and edited manually. The program ALSCRIPT⁵⁰ was used for presentation of the sequence alignment.

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